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# The synthesis of enzyme-bound ATP by the $F_1$-ATPase from the thermophilic bacterium PS3 in the presence of organic solvents

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**Synthesis of enzyme-bound ATP was demonstrated with purified $TF_1$ ($F_1$-ATPase from thermophilic bacterium PS3) from medium inorganic phosphate ($P_i$) and enzyme-bound ADP in the presence of organic solvents such as dioxane, ethanol, dimethylformamide, methanol, acetone, acetonitrile or ethyleneglycol. The optimal concentrations of dimethylformamide, ethanol or methanol were 50%, 30% and 40% and the half-maximal concentrations of $P_i$ were 13 mM, 20 mM and 18 mM, respectively. Thus it is evident that the effect of dimethylsulfoxide on $TF_1$ to form enzyme-bound ATP [8] is not due to a specific interaction between dimethylsulfoxide and the enzyme, but to a decrease in polarity of the medium. In the presence of methanol, the dependence of ATP synthesis on various divalent metal ions was compared to that for the ATP-hydrolyzing activity and the ATP-driven proton-translocating activity of $TF_1$. While $Mn^{2+}$, $Co^{2+}$, $Zn^{2+}$ and $Cd^{2+}$ are as effective as $Mg^{2+}$ for the ATP-hydrolyzing activity of $TF_1$, $Zn^{2+}$ and $Cd^{2+}$ are either less or not effective for proton translocation and for ATP synthesis. This result appears to be consistent with the idea that the $TF_1$-ATP complex formed in organic solvents represents one of the intermediates in the reaction sequences of ATP synthesis by $H^+$-ATPase using the proton gradient.**

## Introduction

$F_1$-ATPase is the catalytic moiety of the $H^+$-ATPase which catalyzes ATP synthesis from ADP and $P_i$ using the electrochemical potential difference of proton across the mitochondrial inner membranes, chloroplast thylakoid membranes and bacterial plasma membranes [1]. ATP synthesis by purified $H^+$-ATPase can be observed using reconstituted vesicles [2–4]. However, such a system is too complicated to study the mechanism of ATP synthesis. Penefsky et al. have studied the mechanism of ATP hydrolysis by the soluble bovine heart mitochondrial $F_1$-ATPase using rapid-mixing methods [5]. Their results show that the equilibrium constant of ATP hydrolysis at a single site is about 0.5 and that the binding constant of ATP to this site is very large (about $10^{12}$ $M^{-1}$). This suggests that during ATP synthesis the electrochemical potential difference is required not for the formation of the covalent bond, but for the release of produced ATP from the enzyme. Feld-

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Abbreviations: $TF_1$, $F_1$-ATPase from thermophilic bacterium PS3; $MF_1$, $F_1$-ATPase from mitochondria; $CF_1$, $F_1$-ATPase from chloroplast; DMSO, dimethylsulfoxide; $P_i$, inorganic phosphate; CDTA, 1,2-cyclohexanediaminetetraacetic acid; Mes, 4-morpholineethanesulfonic acid; ANS, 8-aminonaphthalene 1-sulfate;

man and Sigman [6] reported that enzyme-bound ATP was synthesized by $CF_1$ (chloroplast $F_1$-ATPase) from medium $P_i$ and enzyme-bound ADP when the concentration of $P_i$ was very large. Sakamoto succeeded in synthesizing enzyme-bound ATP by $MF_1$ (mitochondrial $F_1$-ATPase) from medium $P_i$ and ADP in the presence of DMSO (dimethylsulfoxide) at rather low concentration of $P_i$ [7]. Yoshida has also reported that $TF_1$ ($F_1$-ATPase of a thermophilic bacterium PS3) synthesizes enzyme-bound ATP in the presence of DMSO [8]. These results appear to support the result of Penefsky et al. that the equilibrium constant of ATP-hydrolysis on the enzyme is near unity. However, the precise role of DMSO or whether the formed $F_1$-ATP complex corresponds to one of the intermediates of ATP synthesis by the $H^+$-ATPase are not known. Sakamoto has suggested that DMSO affects that the apparent affinity of $F_1$ for $P_i$ and thus by mass-action the step of bound-ATP formation from the enzyme-ADP-$P_i$ complex in the ATP synthesis reaction [9]. In this report we show that several kinds of organic solvents, such as dioxane, ethanol, dimethylformamide, methanol, acetone, acetonitrile and ethyleneglycol have similar effects to DMSO on the synthesis of enzyme-bound ATP and that the divalent metal ions requirement for ATP synthesis in methanol resembles that for proton-translocating activity catalyzed by the $H^+$-ATPase.

## Materials and Methods

*Materials.* ATP-Tris salt was the product of Boehringer. Other adenine nucleotides used were of the highest purity available from Sigma. In order to remove contamination of ATP, ADP was applied to Dowex $1 \times 4$ column ($0.7 \times 2$ cm) equilibrated with 60 mM HCl, eluted from the column and neutralized by 1 M NaOH. The concentration of ADP was determined by the absorbance at 259 nm. The amount of contaminating ATP, measured by a luciferine-luciferase assay [10], decreased from 0.6% of ADP to less than 0.001%. Mes was purchased from Sigma. CDTA, dimethylformamide and DMSO were from Wako Pure Chemical Company. The divalent metal ion salts were of the highest purity available from Wako Pure Chemical Company. Methanol and acetonitrile of the special grade for liquid chromatography were purchased from Merck. All other chemicals were the products of Kanto Chemical Co., Inc.

*Preparation of ADP-loaded $TF_1$.* $TF_1$ was prepared from the PS3 plasma membrane as described in Ref. 11, lyophilyzed after dialysis against a excess amount of pure water and stored at $-80$°C. The lyophilyzed $TF_1$ was dissolved in 50 mM triethanolamine buffer (triethanolamine sulfate (pH 7.3) containing 10 $\mu$M CDTA). ADP and $MgSO_4$ were added to the solution to final concentrations of 2 mM and 5 mM, respectively. After 2 h incubation at room temperature, unbound ADP and $Mg^{2+}$ were removed by passage through a Sephadex G-50 centrifuge column equilibrated with 50 mM triethanolamine sulfate (pH 7.3) containing 100 $\mu$M CDTA [12]. Purified $TF_1$ contains very little, if any, bound adenine nucleotide, but ADP-loaded $TF_1$ has about 3 mol ADP per mol $TF_1$.

*ATP synthesis reaction.* ATP synthesis was started by the addition of 10 $\mu$l of ADP-loaded $TF_1$ to 40 $\mu$l of a mixture containing the other components. Unless otherwise specified in the figures and tables the final mixture contains 1–3 mg/ml of ADP-loaded $TF_1$, 10 mM triethanolamine sulfate, 2 $\mu$M CDTA, 2 mM $MgSO_4$, 40 mM sodium phosphate (pH 6) and the organic solvent. After 2 h incubation at room temperature, the mixture was applied to Sephadex G-50 centrifuge column equilibrated with triethanolamine sulfate (pH 7.3) containing 100 $\mu$M CDTA. The volume of effluent was adjusted to 120 $\mu$l by the addition of triethanolamine buffer and 20 $\mu$l was removed for protein assay. Bound nucleotides were extracted from the protein by the addition of 4 $\mu$l of 60% perchloric acid. The denatured protein was removed by centrifugation (10000 r.p.m., 5 min) and 5 $\mu$l 5 M $K_2CO_3$ was added. The $KClO_4$ formed was removed by centrifugation (10000 r.p.m., 5 min) and the supernatant was used for quantitative analysis of adenine nucleotides by HPLC. In the experiment where the divalent metal ion requirements were assessed, ATP synthesis was started by the addition of 10 $\mu$l $TF_1$ dissolved in triethanolamine buffer to 40 $\mu$l of mixture containing other components to give a final concentration of 1–3 mg/ml $TF_1$, 2 mM ADP, 5 mM divalent metal ion, 40 mM sodium phosphate

(pH 6), and 40% methanol. In order to remove the possibility of ATP contamination in ADP, a control was made replacing sodium phosphate (pH 6) by Mes-Na (pH 6).

*HPLC analysis of adenine nucleotide.* Adenine nucleotide solutions were chromatographed on a Toyo-Soda IEX-540 column (4 × 300 mm) with a Waters model 204 liquid chromatograph as described in Ref. 13. The column was equilibrated with 0.4 M sodium phosphate, (pH 6), (Tables I and II, Figs. 1 and 4a) or 30% methanol in 0.1 M sodium phosphate (pH 7) (Figs. 2, 3, 4b and 4c) and elution was carried out in the same buffer. Nucleotides were monitored by the absorbance at 260 nm and peak areas were determined by automatic integration. The flow rate was 0.8 ml/min and 10 pmol nucleotide were easily analyzed in 10 min using 0.4 M $NaP_i$ or 30 min using 0.1 M $NaP_i$ in 30% methanol. The ratio of nucleotides to $TF_1$ were calculated assuming the molecular weight of $TF_1$ to be 380000 [11].

*Preparation of $H^+$-ATPase vesicles and assay of proton translocation.* The $H^+$-ATPase complex from the thermophilic bacterium PS3 and soybean phospholipids were prepared as described in Ref. 14. Vesicles were reconstituted from the $H^+$-ATPase and phospholipids by the dialysis method as described in Ref. 15. Proton translocation was measured by the fluorescence of ANS. ANS fluorescence was measured with a Hitachi fluorescence meter model 650-60, using an excitation wavelength of 365 nm and measuring emission in cuvette containing 2.0 ml of 50 mM Tris-sulfate (pH 8), 2 mM divalent metal ion and 10 ng of ANS at 40°C. After 10–15 min the reaction was initiated by adding 100 μl of 0.3 M ATP-Tris (pH 8). The fluorescence was enhanced by the proton translocation.

*Other methods.* ATP hydrolysis was assayed as described in Ref. 16, except the temperature of the assay was 40°C instead of 60°C. Specific activity of $TF_1$ in this condition was 2 unit/mg. Protein concentrations were determined with Coomassie brilliant blue G-250 as described by Bradford [17] using $TF_1$ solution prepared from lyophilized $TF_1$ as standards for each experiment.

## Results and Discussion

*The synthesis of enzyme-bound ATP in the presence of organic solvents*

Table I shows the synthesis of enzyme-bound ATP in the presence of organic solvents. These organic solvents satisfy the following two conditions: (1) they are miscible with water; (2) $TF_1$ is not denatured in 20% (v/v) aqueous solutions of these solvents. Because of the high concentration of $P_i$ (40 mM), small amounts of enzyme-bound ATP (less than 0.02 mol per mol $TF_1$) were synthesized in the absence of organic solvent. However, the yield of enzyme-bound ATP exceeded 0.1 mol per mol $TF_1$ in 20% of dioxane or ethanol, in 40% of dioxane or ethanol $TF_1$ was almost completely denatured and precipitated. In DMSO, dimethylformamide, methanol or acetonitrile the amount of enzyme-bound ATP synthesized increased as the concentration of organic solvent increased from 20% to 40%. Although 20% ethyleneglycol had no effect on the synthesis of enzyme-bound ATP, in 40% of ethyleneglycol synthesis of 0.06 mol enzyme-bound ATP per mol $TF_1$ was observed. Glycerol was likely to have no

TABLE I

THE SYNTHESIS OF ENZYME-BOUND ATP IN THE PRESENCE OF ORGANIC SOLVENTS

Experimental conditions are described in Materials and Methods.

| Organic solvent | ATP/$TF_1$ | |
|---|---|---|
| | Concentration of organic solent | |
| | (20%) | (40%) |
| Water [a] | 0.02 | – |
| DMSO | 0.06 | 0.55 [b] |
| Dioxane | 0.18 | 0.00 [c] |
| Ethanol | 0.14 | 0.04 [c] |
| Dimethylformamide | 0.10 | 0.34 |
| Methanol | 0.08 | 0.32 |
| Acetone | 0.07 | not done |
| Acetonitrile | 0.06 | 0.16 |
| Ethyleneglycol | 0.02 | 0.06 |
| Glycerol | 0.02 | 0.02 |

[a] Small amount of enzyme-bound ATP is synthesized without addition of organic solvents because of high concentration of $P_i$.

[b] The concentration of DMSO was 45%.

[c] $TF_1$ was denatured and precipitated.

effect on ATP synthesis. Acetone was as effective as methanol or acetonitrile in allowing ATP synthesis. These results clearly show that the synthesis of enzyme-bound ATP in DMSO is not due to a specific interaction between DMSO and $TF_1$, but to a decrease of polarity of the medium. A decrease of polarity generally causes the increase of activity of hydrophilic materials. It is reasonable, therefore, to suggest that the activity of the phosphate ion increases with the addition of organic solvent, resulting in the increase of affinity of $P_i$ for $F_1$. This agrees with the observation that DMSO increases the apparent affinity of $P_i$ for $F_1$ [9]. The increase of affinity of $P_i$ for $Ca^{2+}$-ATPase in DMSO can also be explained this way [18]. We should add another possibility that the change of polarity in the medium induces some conformational changes in $F_1$, which are preferable for ATP synthesis.

*The dependence of the synthesis of enzyme-bound ATP and ATPase activity on the concentration of organic solvents*

Fig. 1 shows the dependence of the amount of enzyme-bound ATP synthesized and ATPase activity on ethanol concentration. The amount of enzyme-bound ATP gradually increases to 30% ethanol and then decreases with further increase of ethanol concentration. ATPase activity sharply increases and reaches a maximal value of about 8 times the original activity at 24% ethanol. $TF_1$ begins to precipitate in 40% ethanol and completely precipitates in 50% ethanol. The amount of enzyme-bound ADP gradually decreases with the increase of ethanol concentration and the sum of enzyme-bound ADP plus ATP was constant at about 1.3 mol per mol $TF_1$ under 30%. Fig. 2 shows the influence of dimethylformamide concentration on the synthesis of enzyme-bound ATP and ATPase activity. The yield of ATP increases as dimethylformamide concentration increases from 0% to 50%. In 60% dimethylformamide the reaction mixture becomes turbid and in 70% dimethylformamide $TF_1$ was completely denatured and precipitated. ATPase activity increases with the increase of dimethylformamide concentration and a maximal level was maintained between 40% and 50% of dimethylformamide. The amount of enzyme-bound ADP decreases with increased dimethylformamide concentration and the sum of enzyme-bound ADP and ATP was about 1.0 under 40%. Although the yield of enzyme-bound ATP was maintained at about 0.4 mol per mol $TF_1$ in 50% dimethylformamide, the amount of enzyme-bound ADP decreases to 0.1. The dependence of the yield of enzyme-bound ATP and ATPase activity on methanol concentration is shown in Fig. 3. The yield of enzyme-bound ATP increases until 40% and maximal level was maintained between 40% and 50% methanol. In 60%

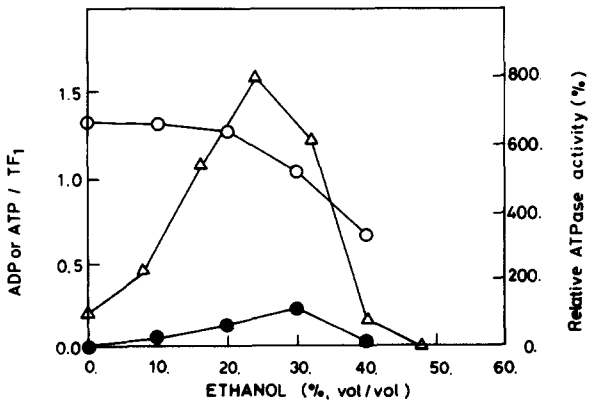



Fig. 1. Dependency of ATP synthesis on ethanol concentration. Experimental conditions are described in Materials and Methods. ●, enzyme-bound ATP; ○, enzyme-bound ADP; △, relative ATPase activity.

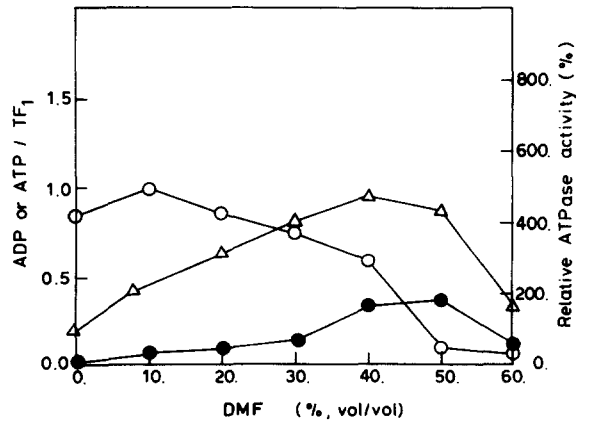



Fig. 2. Dependency of ATP synthesis on dimethylformamide concentration. Experimental conditions are described in Materials and Methods. ●, enzyme-bound ATP; ○, enzyme-bound ADP; △, relative ATPase activity.

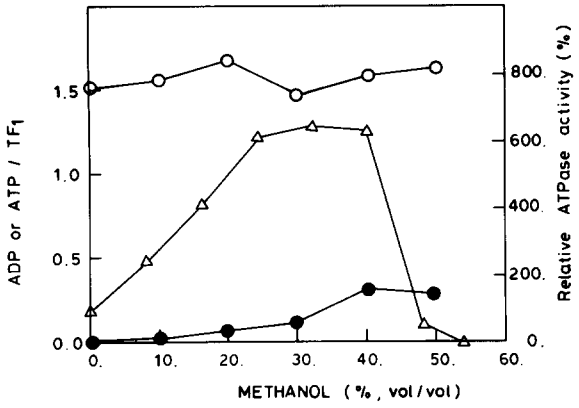
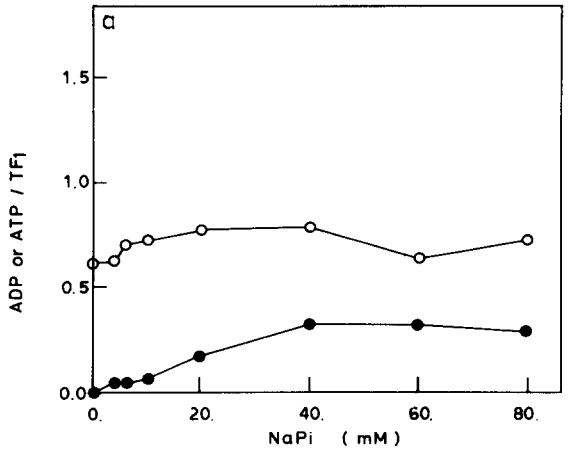



Fig. 3. Dependency of ATP synthesis on methanol concentration. Experimental conditions are described in Materials and Methods. ●, enzyme-bound ATP; ○, enzyme-bound ADP; △, relative ATPase activity.

methanol $TF_1$ was completely denatured and precipitated. Maximal ATPase activity was obtained between 24% and 40% methanol and then it decreases with the increase of methanol concentration. Whereas most of ATPase activity was lost in 50% methanol, the amount of enzyme-bound ATP was kept at the maximal level in the same condition. The amount of enzyme-bound ADP was not affected by methanol concentration. Thus these results show that the yield of enzyme-bound ATP increases with the increase of the concentration of organic solvents, that is, the decrease of polarity of the medium, until $TF_1$ begins to denaturate. The sum of enzyme-bound ATP and ADP synthesized in ethanol and dimethylformamide was nearly constant until $TF_1$ begins to denature as observed with DMSO [8]. But the sum of enzyme-bound ADP and ATP in methanol increases as the concentration increases. ATPase activity was significantly enhanced by all of these three organic solvents.



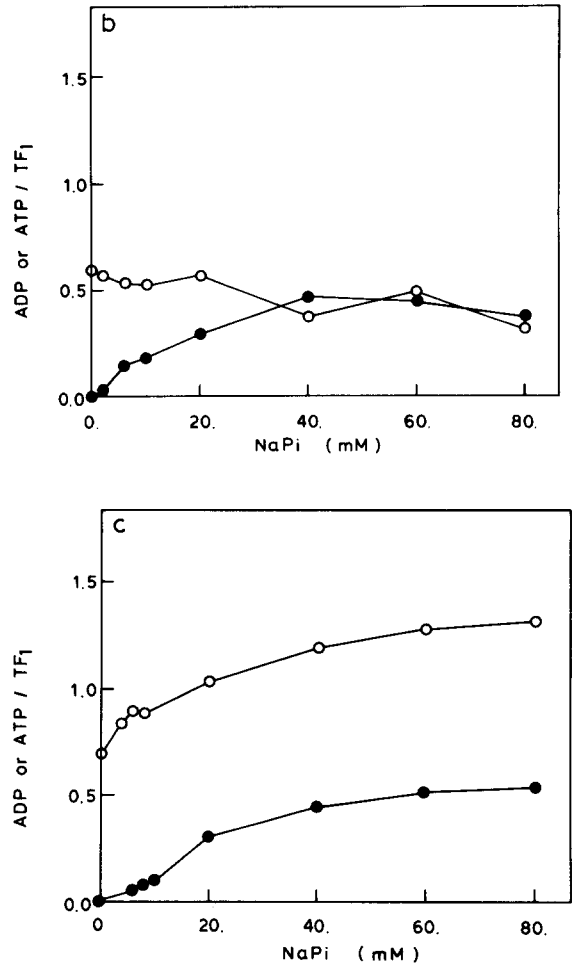




Fig. 4. Dependence of ATP synthesis in the presence of organic solvents on $P_i$ concentration. The reaction mixture contained 40 mM Mes-NaOH (pH 6.0), 2 mM $MgSO_4$, ADP-loaded $TF_1$, various concentration of sodium phosphate buffer (pH 6.0) and (a) 30% ethanol, (b) 50% dimethylformamide or (c) 40% methanol. Other conditions are described in Materials and Methods. ●, enzyme-bound ATP; ○, enzyme-bound ADP.

*Dependence of ATP synthesis on $P_i$ concentration*

The dependence of the yield of enzyme-bound ATP synthesized in the presence of ethanol, di-

methylformamide and methanol on $P_i$ concentration are shown in Fig. 4. The amount of enzyme-bound ATP synthesized in the presence of organic solvents increases with the increase of $P_i$ concentration. The half maximal concentration of $P_i$ for ATP synthesis in ethanol, dimethylformamide and methanol was estimated to be 13 mM, 20 mM and 18 mM, respectively. These values are much larger than that found in DMSO (1 mM) [8]. The sum of enzyme-bound ADP and ATP increases with the increase of $P_i$ concentration. This suggests that the binding of adenine nucleotide (ADP and ATP) to $TF_1$ is also affected by $P_i$.

*The divalent metal ion specificity for ATP synthesis in the presence of organic solvents, ATPase activity and ATP-driven proton-translocating activity*

Table II shows the metal-ion dependency of ATPase activity, ATP-driven proton-translocating activity and ATP synthesis of $TF_1$ in the presence of methanol. Although $Mn^{2+}$, $Zn^{2+}$, $Co^{2+}$ and $Cd^{2+}$ are all as effective as $Mg^{2+}$ for ATPase activity, $Zn^{2+}$ and $Cd^{2+}$ have no effect on the synthesis of enzyme-bound ATP and are less effective for ATP-driven proton-translocating activity. These results appear to support the notion that the ATP-$TF_1$ complex formed in the presence of methanol is one of the intermediates of ATP synthesis by the $H^+$-ATPase using the electrochemical potential difference of proton across the membrane.

TABLE II

DIVALENT METAL ION DEPENDENCY OF ATPASE ACTIVITY OF $TF_1$, ATP-DRIVEN PROTON-TRANSLOCATING ACTIVITY OF $H^+$-ATPASE AND ATP SYNTHESIS BY $TF_1$ IN THE PRESENCE OF METHANOL

The data of the divalent metal ion dependency of ATPase activity are cited from Table II of Ref. 19. Divalent metal ion concentration was 0.2 mM, 2 mM or 0.5 mM for measuring ATPase activity, proton-translocating activity or ATP synthesis, respectively. Experimental conditions for measuring the divalent metal ion dependency of ATP-driven proton translocating activity are described in Materials and Methods. The methanol concentration was 40% for ATP synthesis. Other conditions for measuring divalent metal ion dependency for ATP synthesis are also described in Materials and Methods. No ATP was synthesized without addition of $P_i$.

| Divalent metal ion | ATPase activity (%) | Proton-translocating activity (%) | Synthesis of enzyme-bound ATP (ATP/$TF_1$) |
|---|---|---|---|
| $Zn^{2+}$ | 107 | 15 | 0.02 |
| $Mn^{2+}$ | 104 | 77 | 0.18 |
| $Co^{2+}$ | 102 | 77 | 0.22 |
| $Cd^{2+}$ | 102 | 29 | 0.02 |
| $Mg^{2+}$ | 100 | 100 | 0.20 |
| $Ca^{2+}$ | 7 | 7 | 0.02 |

## Concluding remarks

The synthesis of enzyme-bound ATP in the presence of organic solvents by $TF_1$ is due to a decrease in polarity in aqueous solution and the $TF_1$-ATP complex formed appears to be one of the intermediates in the ATP synthesis by the $H^+$-ATPase.

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